

Compartmentalization within the Nucleus: Discovery of a Novel Subnuclear Region

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Abstract. Antibodies to a set of structurally related autoantigens (p23–25) bind to a previously uncharacterized, large structural domain in the nucleus of a variety of human cell types. This subnuclear domain is visible by phase contrast alone as a region of decreased density after several different fixation protocols. The morphology of this region changes dramatically during the cell cycle and we have given it the name PIKA (for polymorphic interphase karyosomal association) based on preliminary evidence that the PIKA proteins may be associated with chromatin. The function of the PIKA is not yet known, but our immunolocalization

data indicate that it is unlikely to be associated with regions of ongoing DNA replication, heterogeneous nuclear RNA storage, or mRNA processing. The discovery of the PIKA provides evidence supporting an emerging model of nuclear structure. It now appears that the nucleus is organized into distinct domains which include not only the nucleolus, but also previously unidentified regions such as the PIKAs. Furthermore, structural rearrangements undergone by the nucleolus and the PIKAs may be indicative of a broad tendency for nuclear organization to change in a cell cycle-specific fashion.

BEYOND such obvious features as the nucleolus, heterochromatin, and the nuclear membrane, the structural organization of the nucleus is surprisingly poorly understood. While many aspects of nuclear physiology have proven amenable to analysis *in vitro*, a complete understanding of the cellular control of nuclear processes, which include transcription, nascent RNA splicing and processing, and transport of mature mRNA, may ultimately depend on our knowledge of the organization of the nucleoplasmic material surrounding the interphase chromosomes. At present, much about the organization of the nucleoplasm remains unclear. Specifically, it is unknown to what extent the nucleoplasm is functionally and structurally partitioned into separate domains.

Efforts to understand the structure of the nucleoplasm by biochemical fractionation have met with only limited success. Many attempts at separating the nucleus into its constituent substructures have involved disruptive procedures such as nuclease digestion and/or detergent and salt extraction (Berezney and Coffey, 1975; Berezney, 1984; Vogelstein et al., 1985). Biochemical fractionation procedures have permitted identification of proteins associated with small nuclear RNA (snRNA)¹ (Lerner and Steitz, 1979; Pettersson

et al., 1984; Yang et al., 1981), heterogeneous nuclear RNA (hnRNA) (Long et al., 1979; vanEekelen and van Venrooij, 1981; Dreyfuss, 1986), and, to some extent, those associated with newly synthesized DNA (Pardoll et al., 1980; Vogelstein et al., 1980; Berezney, 1984) and with the bases of putative chromosomal loop domains (Mirkovitch et al., 1984; Adachi et al., 1989). However, the use of extractive procedures for analysis of the higher-order substructure of the nucleus has remained controversial (Mirkovitch et al., 1984; Lothstein et al., 1985; Cook, 1988).

An alternative method of studying basic nuclear structure is the use of antibodies to study the distribution of various proteins *in situ*. Autoantibodies from patients with autoimmune diseases have been useful in the analysis of the distribution of small nuclear ribonucleoproteins (snRNPs) (Lerner et al., 1981; Spector, 1990) and centromeres (Moroi et al., 1980; Cooke et al., 1990). Other autoantibodies have recently been used to describe a novel "nuclear dot" domain within the nuclei of proliferating cells (Ascoli and Maul, 1991). In the present study, we use a new experimental antiserum to describe a group of antigens with a novel and dynamic nuclear distribution.

This antiserum, which recognizes a family of three or four structurally related nuclear proteins 23–25 kD in size, identifies large, previously undescribed compartments of the nucleoplasm that are visible both after staining with specific antibody and by phase-contrast microscopy. We have termed this newly identified constellation of subnuclear regions the PIKA (for polymorphic interphase karyosomal association).

These results support the notion that the nucleoplasm is to some extent organized into distinct domains. As discussed

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1. *Abbreviations used in this paper:* CENP, centromere protein; hnRNP, heterogeneous nuclear ribonucleoproteins; PCNA, peripheral cell nuclear antigen; PIKA, polymorphic interphase karyosomal association; SnRNP, small nuclear ribonucleoproteins.

below, this putative structural compartmentalization may reflect a functional segregation within the nucleus as well.

Materials and Methods

Immunological Reagents

The autoimmune patients' sera were provided through the generosity of Naomi Rothfield (University of Connecticut, Farmington, CT) and have been characterized in detail elsewhere (Earnshaw and Rothfield, 1985). Purified anti-PCNA IgG was provided courtesy of Sabina Hildebrandt (University of Connecticut) and Naomi Rothfield. The anti-Sm Y12 monoclonal antibody was provided courtesy of Joan Steitz (Yale University, New Haven, CT) and Joe Gall (Carnegie Institution, Baltimore, MD). The anti-hnRNP protein antibodies were a gift from S. Pinol-Roma and G. Dreyfuss (University of Pennsylvania, Philadelphia, PA). The anti-DNA antibody was a gift of David Stollar (Tufts University, Boston, MA).

Cell Lines and Culture Techniques

HeLa cells were grown in RPMI-1640 (Gibco Laboratories, Grand Island, NY) supplemented with 5% HyClone calf serum (HyClone Sterile Systems, Logan, UT). Primary umbilical cord fibroblasts and primary foreskin fibroblasts were gifts of Gail Stetten (Johns Hopkins Medical School, Baltimore, MD). K-562 cells were provided by J. Roberts (Hutchinson Cancer Center, Seattle, WA).

Preparation of Crude Nuclear Extract

Nuclear proteins were isolated from HeLa cells grown in suspension and swollen in RSB (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 5 mM MgCl₂) at room temperature for 5 min. The cells were then lysed at 4°C in 15 mM Tris-HCl, pH 7.4, 80 mM KCl, 2 mM K-EDTA, pH 7.4, 0.75 mM Spermidine, 0.30 mM Spermine. Lysis was accomplished with 10 vigorous strokes with a type A pestle in a 15-ml Dounce homogenizer (Wheaton Scientific, Millville, NJ). The homogenate was centrifuged at 100 g for 15 min and the pellet containing nuclei resuspended in 1% SDS protein sample buffer.

Immunoblotting

The immunoblotting procedure has been described previously (Earnshaw et al., 1984).

Immunofluorescence

Cells were grown on 18-mm² glass coverslips and then fixed as described in the figure legends. The coverslips were washed twice by immersion for 10 s and 5 min in KB buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.7, 0.1% Triton X-100, 0.01% BSA [Pentax grade, Miles Laboratories, Inc., Elkhart, IN, 30% solution]) and then for 5 min in KB without Triton X-100 (KB⁻). The excess buffer was removed from the coverslips by gentle shaking and blotting the edge and back with tissue paper. After this, 75 μl of the following solutions (diluted in KB⁻) were added to the surface of the coverslip in the following order. (a) Primary antibodies diluted as follows: Rabbit anti-PIKA sera 1:1,000; autoimmune anticomere serum 1:10,000; purified IgG anti-PCNA autoantibody 1:1,000; mouse monoclonal anti-Sm culture supernatant 1:10; purified monoclonal IgG anti-CENP-B ascites fluid 1:200; anti-DNA monoclonal culture supernatant 1:2; anti-L protein antibodies 1:250; anti-C₁ and -C₂ monoclonal ascites fluid 4F4 1:5,000; anti-A₁ monoclonal culture supernatant 4B10 was not diluted. Combinations of two of these antibodies were sometimes used as indicated. (b) Biotinylated anti-rabbit immunoglobulin antibody (Vector Laboratories, Inc., Burlingame, CA) diluted 1:1,000. In experiments such as that shown in Fig. 8, the anti-PIKA antibodies could be added at this step with the same results. (c) Streptavidin conjugated with Texas red (Bethesda Research Laboratories, Gaithersburg, MD), diluted 1:1,000. For experiments where a second antibody was used in step a, fluoresceinated anti-human or anti-mouse was also used at this step at a 1:250 dilution. (d) The DNA binding fluorescent dye DAPI at 1 μg/ml in KB.

For steps a-c the coverslips were placed in a humid chamber and incubated at 37°C for 30 min. The DAPI treatment was done at room temperature for 5 min. The coverslips were washed between antibody additions for 10 s and 5 min in KB and 5 min in KB⁻ as above. After the last wash, the coverslips were inverted and mounted with 25% glycerol in PBS containing

150 mM Diazabicyclo-octane (Aldrich Chemical Co., Inc., Milwaukee, WI) used as an antifade agent and sealed at the edges with nail polish.

Molecular Cloning and Production of Antisera

Autoimmune patient's serum was used to screen a human λgt11 expression vector library. cDNAs were isolated and expressed in *Escherichia coli* from the β-galactosidase promoter, or subcloned into pATH11 (Spindler et al., 1984) and expressed as trpE fusion proteins (Saunders, 1990). These cDNA clones will be described in detail elsewhere (Saunders, W. S., and W. C. Earnshaw, manuscript in preparation).

Approximately 20 mg (estimated after Coomassie blue staining by comparison with known standards) of trpE fusion protein that contained ~13 kD of human polypeptide (38% of total fusion protein) were electrophoresed on an SDS-polyacrylamide gel. A narrow band containing the fusion protein was excised and its identity verified by immunoblotting. By comparison to uninduced control cultures the excised band contained >75% of the total protein as fusion protein. The gel band was soaked twice for 30 min in water to remove the SDS, ground up to a fine powder in a mortar and pestle under liquid nitrogen, and transferred to a glass 2.5-ml syringe. 1 ml of PBS was added, followed by 1 ml of Freund's complete adjuvant (Difco Laboratories, Inc., Detroit, MI). The mixture was then sonicated (output setting 4, Branson Sonifier model 200, Branson Sonic Power Co., Danbury, CT) until a fine emulsion was formed, at which point the solution was too viscous to pour from the syringe. At day 0, one male rabbit (6 mo old, Hazleton Research Products, Denver, PA) was injected in multiple locations: subcutaneously on the back, in the dorsal medial subscapular area, intramuscularly in the gluteal region, and into the parietal peritoneum above the abdomen. The animal was boosted with ~10 mg of the same antigen in incomplete Freund's at 30 and 65 d. Blood was drawn passively from the central ear vein 10 d after the second and third injections and allowed to clot for 30 min at 37°C. The clot was centrifuged at 4,200 g for 30 min, the serum drawn off, respun, and the supernatant aliquoted into small volumes, and stored at -70°C.

Affinity Purification

Affinity purification of specific antibodies from proteins immobilized on nitrocellulose filters was performed as described (Earnshaw and Rothfield, 1985).

Cell Synchrony

HeLa cells were grown attached to 150-cm² flasks for 12-36 h to ~80% confluency. Flasks were preshaken by three vigorous blows with the palm of the hand, the cells were spun down, and the media was returned to the flasks. 2 h later the flasks were gently reshaken and the mitotic cells plated on glass coverslips. At various times after shakeoff the cells were examined by immunofluorescence. In some experiments the cells were partly synchronized after shakeoff by being blocked for 9 h with aphidicolin at 2 μg/ml, washed twice, and released with fresh media. The results with and without aphidicolin pretreatment were indistinguishable and therefore were combined in the subsequent analysis. Populations were considered synchronized if >70% of the cells had cytoplasmic bridges after shakeoff, and >85% were positive for PCNA immunostaining during S phase. Only in some experiments could PIKA immunostaining be readily seen in every cell. For the cell synchrony experiments only the cells where PIKA staining could be clearly seen were counted. Each time point represents 100-600 cells counted during three separate experiments. Examinations were performed blind so that the researcher did not know which time point was being counted.

Electron Microscopy

Immunolectron microscopy was performed as described (Cooke et al., 1990). Briefly, cells were grown on coverslips and fixed in situ with 4% formaldehyde in Dulbecco's PBS. Treatment with primary antibodies and subsequent washing was as for immunofluorescence (see above). The cells were then incubated with goat anti-rabbit IgG conjugated to 1 nm gold (Janssen Life Sciences Products, Piscataway, NJ) diluted 1:100 in KB⁻ for 4 h at room temperature. After washes in KB⁻ and D-PBS, the cells were fixed in 2% glutaraldehyde with 0.02% tannic acid in D-PBS and silver enhanced (IntenSe Silver Enhancement kit; Janssen Life Sciences Products). The cells examined without antibody label were rinsed briefly with D-PBS and then fixed with 2% glutaraldehyde in D-PBS plus 2 mg/ml tannic acid

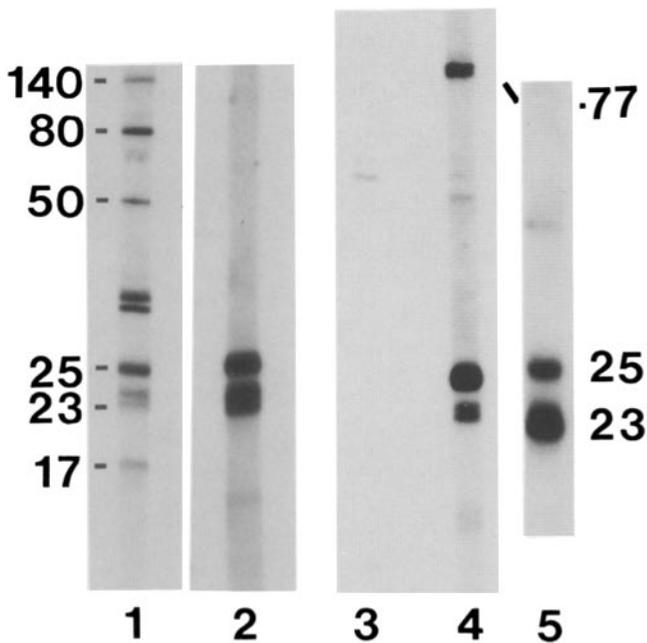


Figure 1. Discovery of antisera recognizing a family of autoantigens of 23–25 kD. HeLa nuclear proteins were electrophoresed on a 15% SDS–polyacrylamide gel and blotted to nitrocellulose. Vertical strips from the blot were probed with serum from an autoimmune patient known to have anticentromere antibodies (lane 1) or autoantibodies affinity purified from a β -galactosidase/p23–25 fusion protein (lane 2). The affinity-purified autoantibodies bind only to the p23–25 kD proteins. The centromere autoantigens at 17 kD (CENP-A), 80 kD (CENP-B), 140 kD (CENP-C), and 50 kD (CENP-D) are indicated. Nuclear proteins were screened with preimmune (lane 3) and immune serum (lane 4) from a rabbit immunized with a trpE/p23–25 fusion protein. The rabbit immune serum, but not the preimmune serum, bound to the p23–25 proteins. The rabbit immune serum also contained antibodies to a cross-reacting protein doublet at \sim 77 kD. Rabbit antibodies affinity purified from the fusion protein immobilized on nitrocellulose bound only to the p23–25 kD proteins on blots (lane 5).

for 30 min. The cells were washed in D-PBS, dehydrated, embedded, and sectioned as described (Cooke et al., 1990).

Nuclear Permeabilization and Enzyme Treatment

HeLa cells were grown overnight on glass coverslips and treated with KBS (KB plus 300 mM sucrose with protease inhibitors) at 4°C for 2–5 min. The protease inhibitor mix included 10 U/ml Trasylol (Mobay Chemical Corp., Pittsburgh, PA), 10 mM PMSF (Sigma Chemical Co. St. Louis, MO), and chymostatin, leupeptin, antipain, and pepstatin (CLAP, Sigma Chemical Co.), each at 1 mg/ml in DMSO. The extracted cells were treated with enzyme (or control buffer without enzyme) for 20 min at 4°C (micrococcal nuclease) or 20 min at room temperature (RNase A or DNase I) in KBS without Triton X-100 (KBS⁻). The buffers for enzymatic digestion were as follows: RNase; KBS⁻, DNase; KBS⁻ plus 10 mM MgCl₂, micrococcal nuclease; KBS⁻ plus 2 mM CaCl₂. The cells were then fixed in methanol at -20°C and processed for immunofluorescence as described above, except the cells were washed in KB⁻ only. DNase and RNase were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN; micrococcal nuclease was from Worthington.

Results

Anti-p23–25 Antibodies

Among those sera from patients with scleroderma spectrum disease that recognize centromeres (Moroi et al., 1980) are

a small number that also recognize a set of autoantigens with molecular masses of \sim 23–25 kD (referred to here as the p23–25 antigens) (Guldner et al., 1984; Earnshaw and Rothfield, 1985). These autoantigens can be seen to form a complex of three to four spots on nonequilibrium two-dimensional gels (Cox et al., 1983; Saunders, W. S., unpublished observations). One such serum (Fig. 1, lane 1) was used to screen a human λ gt11 cDNA library, and clones expressing immunopositive fusion proteins were selected as described elsewhere (Saunders, 1990). Two sorts of clones were obtained. The majority consisted of cDNAs encoding CENP-B, the major centromeric autoantigen (Earnshaw and Rothfield, 1985). Others, which provided the starting point for the experiments described here, encode epitopes present on the p23–25 antigens (Fig. 1, lane 2). Autoantibodies affinity-purified from the fusion protein recognized the entire complex of p23–25 antigens, confirming that these antigens are structurally related (Saunders, W. S., and W. C. Earnshaw, manuscript in preparation).

Polyclonal antibodies to the fusion protein from one such phage clone (that encoded 12.8 kD of human polypeptide) were raised in a rabbit (see Materials and Methods). This immune serum contained antibodies that apparently recognized the entire p23–25 complex of autoantigens (Fig. 1, lane 4). The rabbit immune serum also cross-reacted with several other proteins. These are apparently unrelated to the p23–25 antigens, as shown by affinity purification of antibodies from the rabbit serum using the fusion protein immunogen immobilized on nitrocellulose. Such affinity purified antibodies bind back only to the p23–25 proteins on blots (Fig. 1, lane 5) and also to the PIKAs as described below (see Fig. 3, J and K).

Rabbit Antibodies Bind to a Novel Subnuclear Region

We examined the distribution of the p23–25 proteins in fixed cells by indirect immunofluorescence with the rabbit serum. Antibodies from the immune serum recognized a highly polymorphic set of substructures within HeLa cell nuclei (Fig. 2). We have designated these regions PIKAs.

PIKAs are extremely variable in size and number. They range in size from a single large roughly spherical region (up to 5 μ m in diameter) in the nuclei of some cells to dozens of smaller punctate foci in others. Discrete antibody binding regions are not visible in mitotic and earliest G₁ cells. Instead a low level of diffuse staining is seen throughout the cytoplasm. Preimmune serum from this rabbit recognizes neither the p23–25 antigens (Fig. 1, lane 3) nor the nuclear spots in fluorescence (Fig. 2 B, inset).

Large PIKAs are often quite conspicuous when viewed by phase-contrast microscopy (Fig. 3). They can be recognized as light areas of the nucleus with an irregular, but roughly spherical, shape. PIKAs often appear to have a distinct boundary and may have visible internal structure (Fig. 3, A–C). They also typically appear to be less phase dense than the surrounding nucleoplasm (Fig. 3, D and E). The fluorescent immunolabeling within larger PIKAs has a distinct speckled appearance, suggesting that antibodies may bind to a cluster of subunits within the PIKA region (Fig. 3 E and L). (The speckled appearance is occasionally difficult to see in photographic prints.) In some cells the PIKAs seem to break up into smaller subunits, and the distinct boundary with the rest of the nucleoplasm is lost (see below).

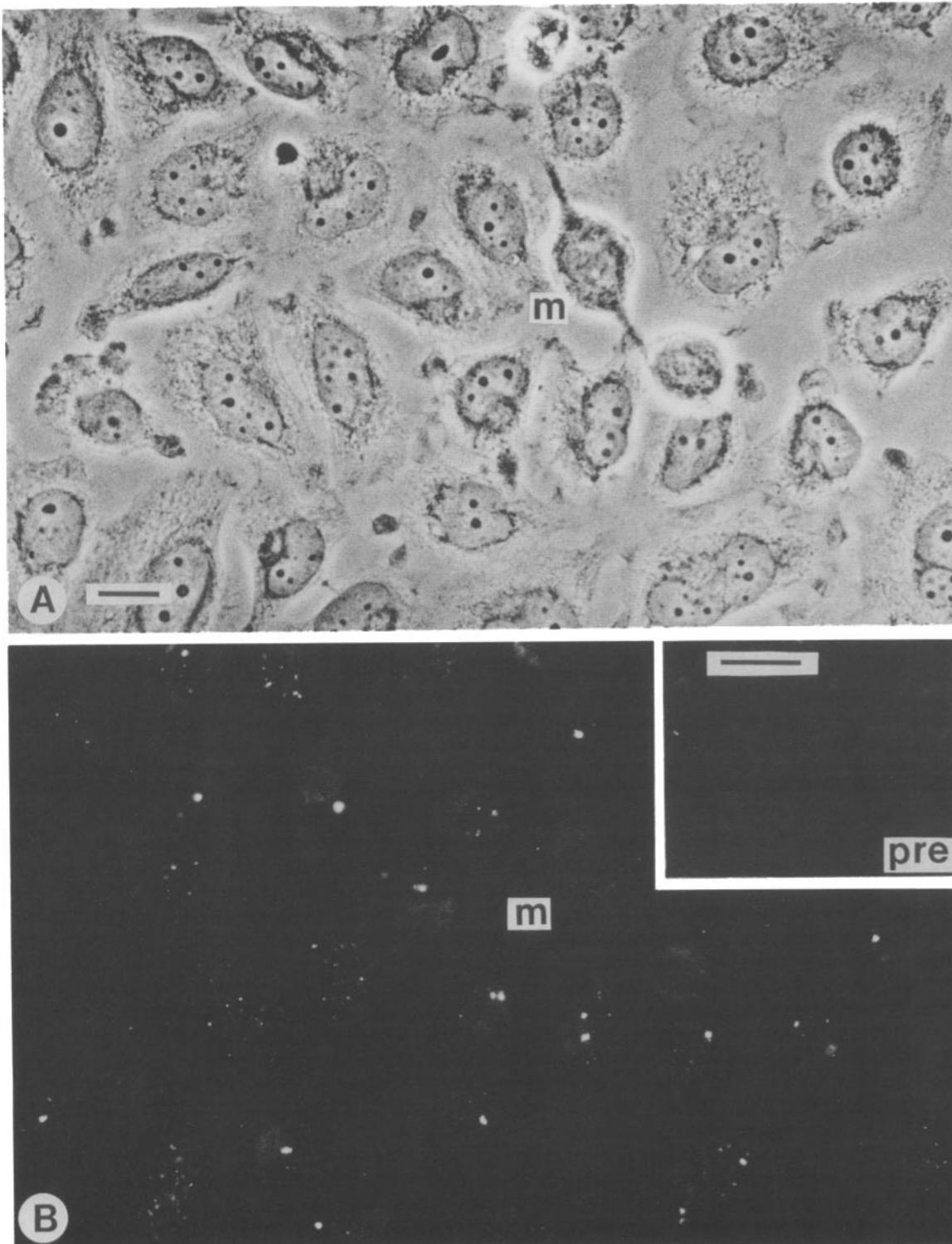


Figure 2. Rabbit antibodies to the 23–25 kD proteins detect a polymorphic population of structures within HeLa cell nuclei. HeLa cells were grown on coverslips, fixed in 100% methanol at -20°C , and screened by indirect immunofluorescence using serum from a rabbit immunized with the p23–25 fusion protein. This antibody recognizes regions within the nucleus of most cells. The size and number of the stained structures varied greatly between different cells. Phase-contrast (*A*) and fluorescence (*B*) images are shown. Preimmune serum was not immunoreactive (*inset* in *B* shows a field containing ~ 25 cells). *m*, mitotic cell. Bars: (*A* and *B*) $20\ \mu\text{m}$; (*inset*) $100\ \mu\text{m}$.

We have also observed other “phase-light” regions of the nucleus that do not react with the anti-PIKA antibodies. These may represent additional nuclear domains similar to the PIKAs, but lacking the p23–25 antigens (Fig. 3, *F* and *G*).

PIKAs Are Present in a Variety of Cell Types

PIKAs appear to be fundamental nuclear features present in a variety of human cell types. They are not specialized features of transformed cells. Epithelial (HeLa), erythroid

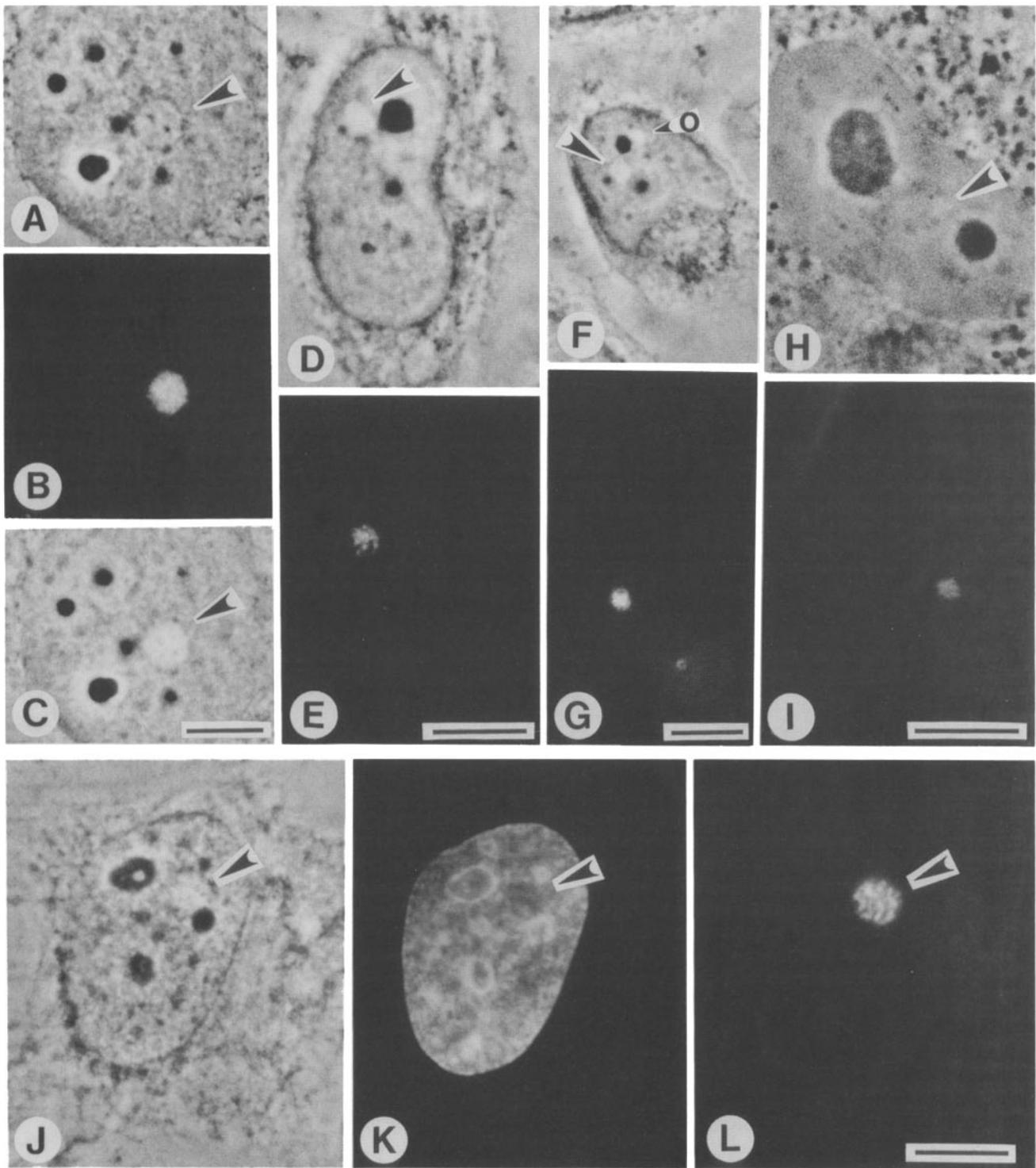
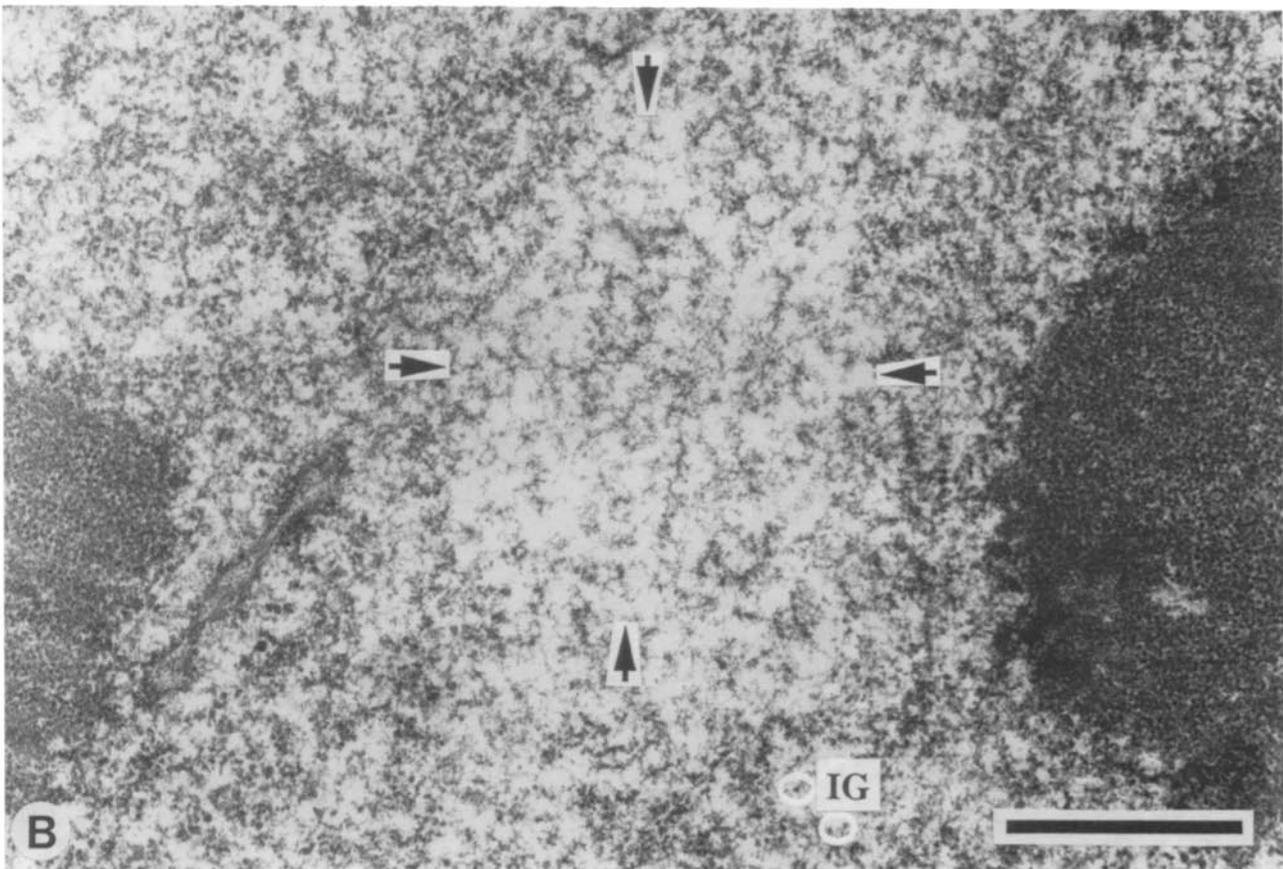
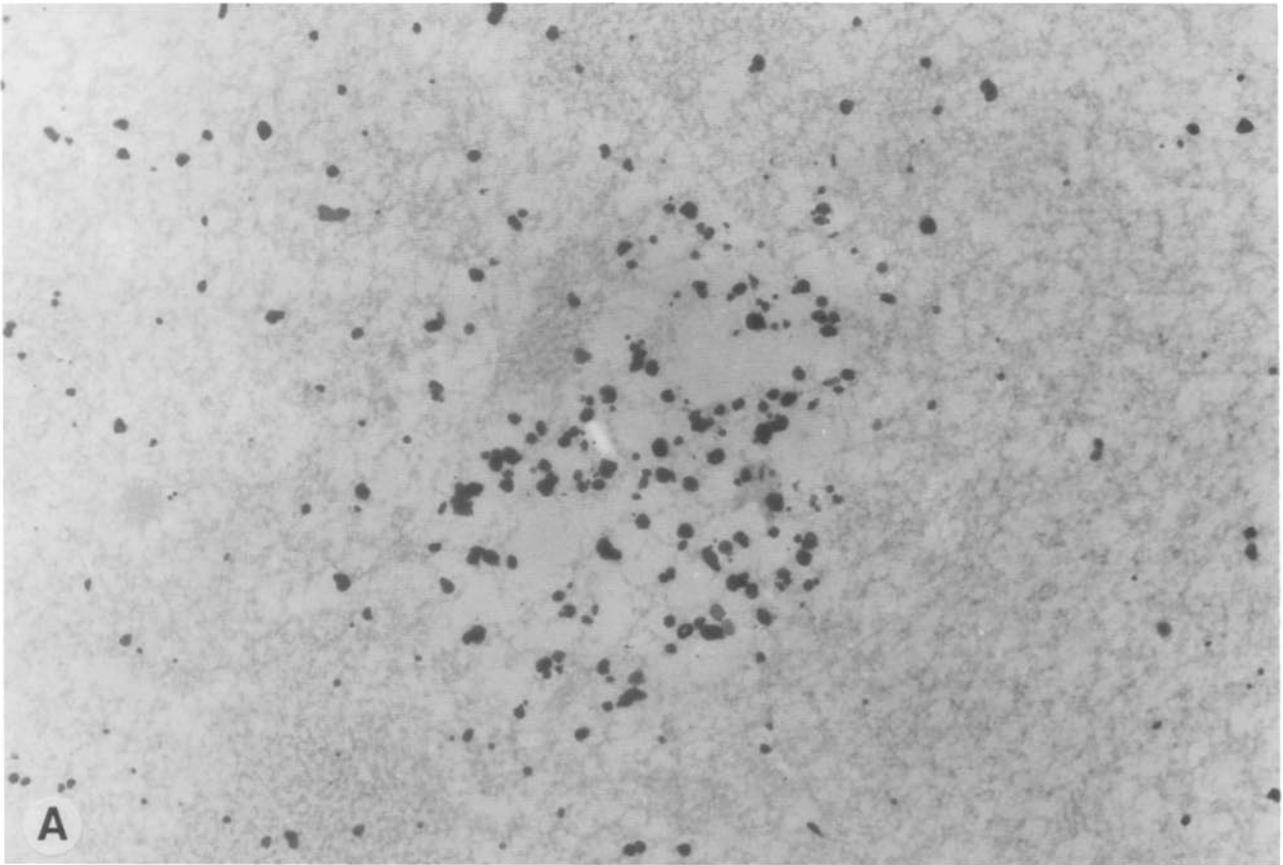


Figure 3. Gallery showing several important aspects of PIKA morphology. (A-C) The PIKA is often directly visible by phase-contrast microscopy. The domains recognized by antibody appear less dense than the surrounding nucleoplasm under phase contrast. (F and G) It is possible to observe phase-light regions of the nucleus that do not react with the anti-PIKA antibodies. In the example shown, a single PIKA is seen in this HeLa cell (F, arrowhead). Another phase-light region of the nucleoplasm was not immunoreactive (F, arrowhead, O). (H and I) Primary human fibroblast nuclei contain PIKAs of morphology similar to those seen in HeLa cells. (J-L) Affinity-purified antibodies specific for the 23–25 kD antigens bind to PIKAs. In this case, antibodies were affinity purified from a bacterial fusion protein (see Fig. 1, lane 5). The granular substructure of the PIKA is particularly visible in L. A, D, F, H, and J show phase-contrast images. B, E, G, I, and L show anti-PIKA fluorescence. Phase and fluorescence images are superimposed in C. DNA staining with DAPI is shown in K. Except for H and I, cells were fixed in 100% methanol at -20°C . The cell in H and I was fixed in 3% formaldehyde in PBS. Bars, 10 μm .



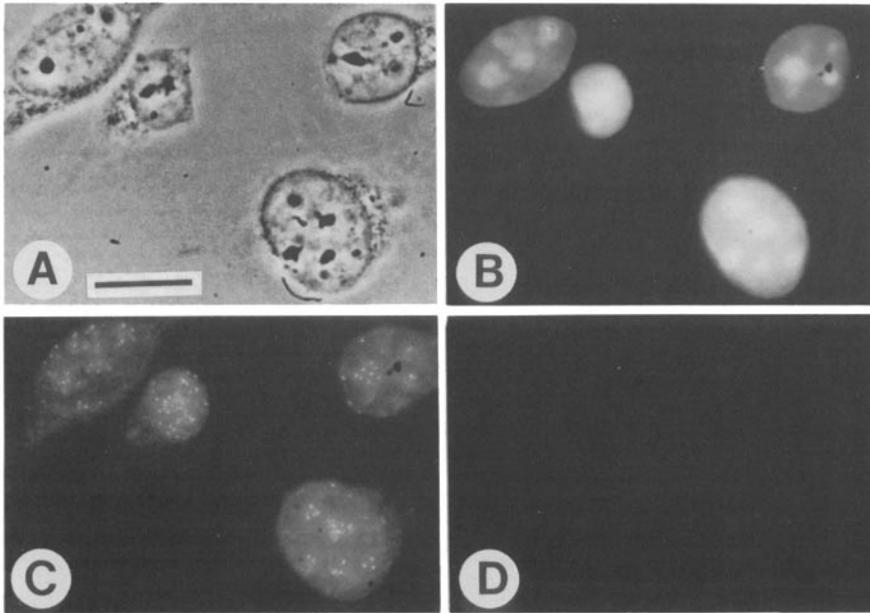


Figure 5. PIKA staining depends on the integrity of the DNA. HeLa cells were permeabilized with a buffer containing Triton X-100 (see Materials and Methods) and treated with micrococcal nuclease before immunofluorescence. (A) Nuclei were still structurally intact as determined by phase-contrast microscopy; (B) DAPI fluorescence was slightly reduced as expected. (C) Centromere immunolabeling was still detected after nuclease treatment. (D) The anti-PIKA staining was greatly reduced or absent (D) from nuclease-treated cells. Cells were fixed in 100% methanol at -20°C . Bar, 20 μm .

(K-562), and two primary diploid human fibroblast cell lines were tested by immunofluorescence with the anti-PIKA serum. PIKAs with a similar distribution in size, shape, and number were observed in all these cell types. An example of such staining of a primary fibroblast is shown in Fig. 3, *H* and *I*. We have not observed specific staining using this serum on cells of other species (including Indian muntjac, BHK, and pig kidney epithelial (LLC-PK) cells although similar phase-light areas have been observed within the cell nuclei.

Anti-p23-25 Antibodies Bind to the PIKAs

Anti-p23-25 antibodies affinity purified from several different substrates bound to PIKAs in a manner qualitatively indistinguishable from that of whole serum. The substrates used for affinity purification included both β -galactosidase and tryptophan synthetase (trp E) p23-25 fusion proteins, as well as gel-purified cellular p23-25 proteins from HeLa cells (Fig. 3, *J* and *K*). When affinity purification experiments were performed with four other unrelated bacterial and human proteins no specific staining was observed (results not shown). These results demonstrate convincingly that antibodies that bound to PIKAs by immunofluorescence also bound to the p23-25 proteins on blots.

The Ultrastructure of the PIKAs

The use of a recently developed procedure for immunolocalization of nuclear antigens with ultrasmall colloidal gold probes (Cooke et al., 1990) permitted us to examine the

structure of the PIKAs by electron microscopy. This procedure revealed that PIKAs have lower electron density than the surrounding nucleoplasm and are traversed by fibrous material with an average diameter of 11-17 nm (Fig. 4 *A*). The rabbit antibody often bound to these fibers at points of intersection. Nonuniform binding may be responsible for the speckled appearance of the PIKAs observed by immunofluorescence (Fig. 3 *E*). The PIKAs were occasionally found near regions of heterochromatin or nucleoli.

Low levels of antibody binding to the cytoplasm and to the rest of the nucleus were also observed, suggesting that the antibody may also react with a diffuse component. No significant binding was seen with preimmune serum (results not shown). Quantitative analysis showed the density of the gold label with the immune serum was >8 times higher in PIKAs than in the rest of the nucleoplasm; >5 times higher than in the cytoplasm; and >38 times higher than in the nucleolus. We do not know whether the low level of background binding in the rest of the nucleus was due to the presence of dispersed PIKA antigens or to nonspecific binding of the gold probes.

When cells are fixed in glutaraldehyde and embedded without antibody treatment, it is possible to identify nuclear regions that resemble PIKAs with regard to size, shape, and distribution. We feel it likely that this is the appearance of the PIKAs in optimally fixed cells (Fig. 4 *B*). The enhanced specimen preservation afforded by glutaraldehyde fixation permits us to observe that these regions are apparently devoid of interchromatin granules, which are nearly ubiquitous in the nucleoplasm (Fakan, 1986). The absence of interchromatin granules could account for the apparent lower

Figure 4. Ultrastructural characterization of the PIKA. (A) PIKAs were detected by preembedding immunoelectron microscopy with rabbit anti-PIKA antibodies. Immunogold label was detected in nuclear regions with lower apparent density than the surrounding nucleoplasm. Fibers traversing the labeled regions appeared to be the sites of antibody binding. Lower levels of immunolabeling could also be detected in other areas of the nucleus, possibly due to a background diffuse distribution of the antigen. (B) HeLa cells were fixed in glutaraldehyde without immunolabeling (see Materials and Methods) and examined for the presence of PIKA-like regions. Putative PIKAs were visible as regions of low electron density in the nucleoplasm (arrows). Interchromatin granules (*IG*, circles) were not detected in these regions. Densely staining areas on either side of the photograph are nucleoli. Bar, 1 μm .

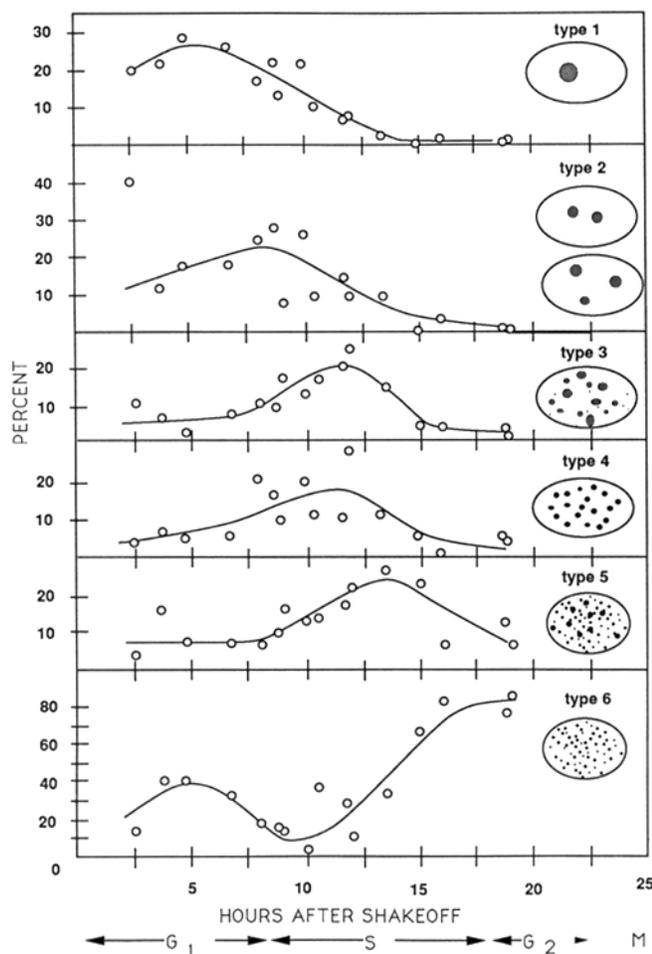


Figure 6. The morphology of the PIKAs changes as a result of cell cycle phase. The frequency of six different types of PIKA structure was determined for synchronized cells at various times after mitotic shake off. Graphs showing the frequency of the PIKAs in synchronized populations are given. The frequency of each type of PIKA changed gradually during the cell cycle. The larger type 1 and type 2 PIKAs were present only in G₁ and early S phase. The smaller types were the most common form later in the cell cycle. These data suggest a gradual shift to smaller and dispersed PIKAs during the cell cycle.

density of the PIKAs when compared to the rest of the nucleoplasm.

PIKAs Requires Intact DNA to Maintain Antigenic Identity

To begin to examine the relationship between PIKAs and DNA, we tested whether enzymatic digestion of the DNA had any effect on PIKA morphology in permeabilized HeLa cells. Cells were permeabilized with a buffer containing 0.5% Triton X-100 and treated with micrococcal nuclease or DNase I before antibody incubation (Fig. 5). These mild digestion conditions reduced, but did not abolish, DNA staining with DAPI (Fig. 5 B). In contrast, the antibody binding to the PIKAs was greatly reduced or absent after nuclease treatment (Fig. 5 D). Abolition of PIKA staining required active enzyme, for no reduction of antibody binding was observed if cells were pretreated with buffer minus nuclease or with micrococcal nuclease without the required

calcium. Not all nuclear substructure was eliminated by the nuclease treatment as shown by phase-contrast examination and by the persistence of centromere staining (Fig. 5 C). Although this experiment should not be taken as proof that DNA is an integral component of the PIKA, the results suggest that PIKAs are associated with DNA at some level. In contrast, RNA integrity is apparently not required to maintain the structure of the PIKAs. PIKA staining was unaffected by treatment with 50 μ g/ml RNase A (results not shown).

We examined the distribution of DNA in the nucleus relative to the PIKAs by immunofluorescence and confocal microscopy. Anti-DNA antibodies bound throughout the nucleus with variable intensity. The anti-PIKA antibodies bound reproducibly to regions of the nucleus with relatively less anti-DNA antibody binding (Fig. 3, K and L). This result was seen regardless of the order in which the anti-PIKA and anti-DNA antibodies were added. This suggests that PIKAs may contain DNA that is less compacted than other areas of the nucleus.

The Morphology of PIKAs Changes during the Cell Cycle

PIKAs undergo a striking change in appearance as HeLa cells traverse the cell cycle. To quantify this phenomena, the morphology of the PIKAs was classified into six categories, and the percentage of cells with each category of PIKA staining was determined for synchronized cells at different times after mitosis (Fig. 6). The large type 1 and type 2 structures were detected only during G₁ and early S phase. As the cycle progressed, the PIKAs gradually became smaller and more numerous, as if the larger PIKAs disassociated into smaller subunits. By late S phase, nearly all cells in the synchronized population had a dispersed punctate nuclear staining (type 6). Micrographs of cells with representative PIKA types are shown in Fig. 7.

Relationship to Other Nuclear Components

The PIKAs appear to be independent from sites of DNA replication and/or concentrations of PCNA, a cofactor of DNA polymerase δ (Bravo et al., 1987; Prelich et al., 1987). HeLa cells were screened simultaneously with anti-PIKA antibodies and anti-PCNA antibodies. The PIKA staining appeared to be independent of the position of PCNA (Fig. 8, A and B). Since the PIKAs were also detected when no DNA synthesis is occurring in the cell (as defined by the absence of PCNA immunofluorescence and in synchronized cells known to be in G₁), we believe the structural differentiation of this nuclear region is not directly due to DNA replication.

Similarly, we examined the distribution of RNA polymerase II in HeLa nuclei relative to the PIKAs. Polymerase II was found to be distributed throughout the nucleus, including the PIKAs. However, it showed no tendency to be concentrated in the PIKA regions (results not shown).

The speckled PIKA staining observed during late S and G₂ phases of the cell cycle resembles that of centromeres (Moroi et al., 1980). However, centromeres and PIKAs are distinct entities. The speckles stained with the anti-PIKA antiserum do not colocalize with antibodies to the centromere-associated CENP-B antigen (Fig. 8, C and D). The confocal

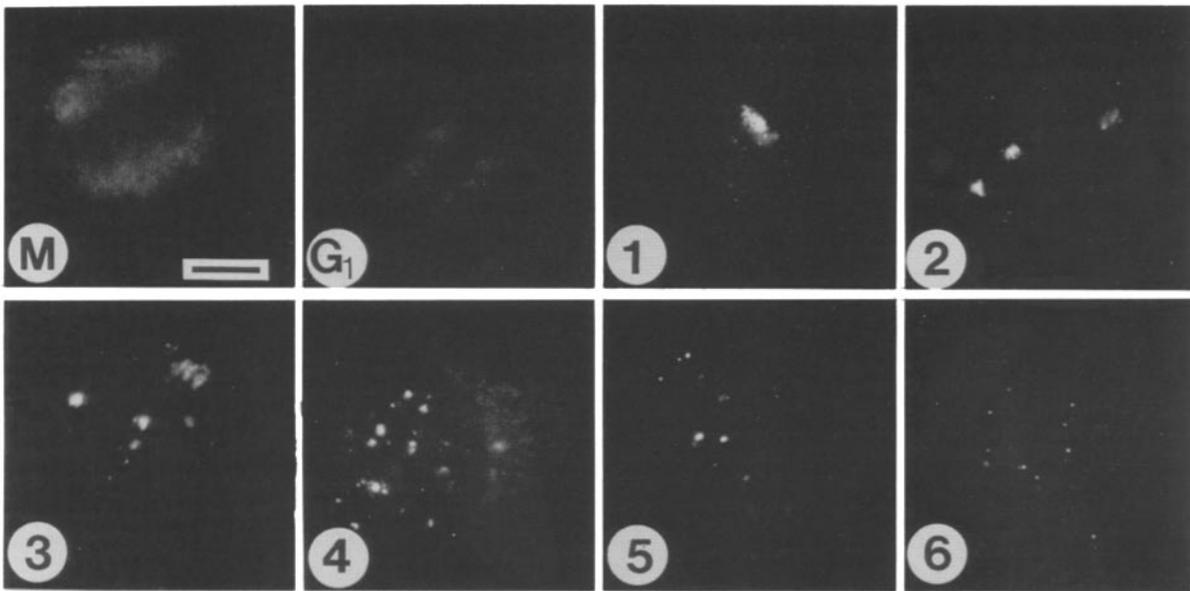


Figure 7. Examples of the six classes of PIKA morphology. During mitosis (M) and very early G₁, only a diffuse cytoplasmic staining can be detected. Numbers 1-6 refer to the morphological classes presented in Fig. 6. Cells were fixed in 100% methanol at -20°C. Bar, 5 μm.

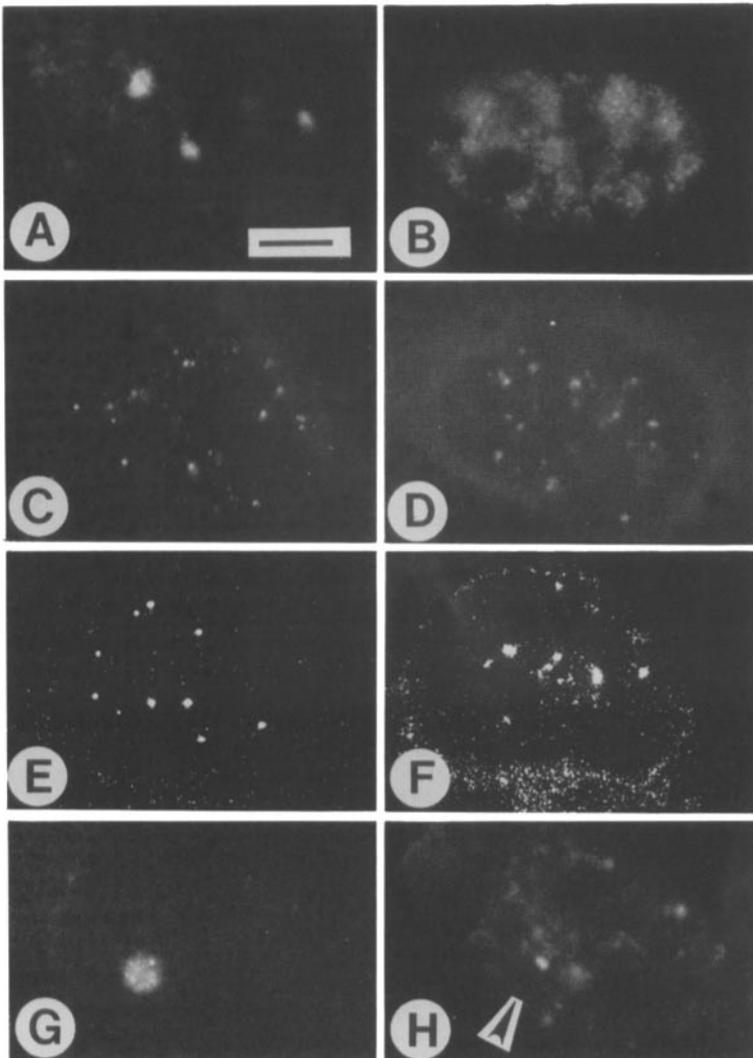


Figure 8. PIKAs do not correspond to regions of ongoing replication, centromeres, or concentrations of snRNP antigens. Anti-PIKA serum immunofluorescence is shown in A, C, E, and G. B shows regions of ongoing DNA replication detected with anti-PCNA. D and F show the location of the centromeric heterochromatin, detected with monoclonal antibody to CENP-B (Earnshaw et al., 1987). (E and F were obtained with the laser scanning confocal microscope.) H shows presumed regions of RNA splicing, detected with monoclonal antibody to the Sm antigens. The arrowhead in H shows PIKA region. PCNA, centromeres, and SM antigens are all apparently absent from the PIKAs. Cells were fixed in 100% methanol at -20°C. Bar, 10 μm.

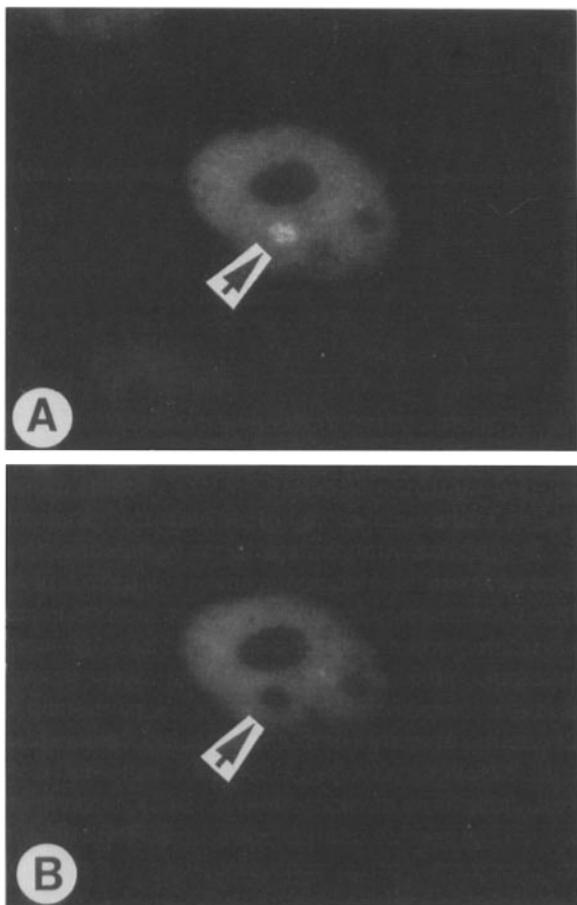


Figure 9. Exclusion of the hnRNP C₁ and C₂ proteins from the PIKA regions. *A* shows the pattern of binding of anti-PIKA antibody to HeLa nuclei. *B* shows the binding of antibody recognizing the hnRNP C₁ and C₂ proteins to the same nucleus. Arrows indicate the PIKA region. C₁ and C₂ proteins are located throughout much of the nucleoplasm but are absent from the PIKA regions. Other regions of decreased antibody binding common to both panels are nucleoli. To emphasize the absence of the hnRNP C₁ and C₂ proteins from the PIKA, *A* was exposed for considerably longer than the other figures showing PIKA staining. Cells were fixed in 100% methanol at -20°C followed by acetone at -20°C for 30 s.

microscopy images of Fig. 8, *E* and *F*, shows the independent distribution of PIKAs and centromeres particularly clearly.

PIKAs are apparently not associated with proteins involved in RNA processing or packaging. The Sm antigens (involved in RNA processing; Lerner et al., 1981) (Fig. 8, *G* and *H*) and the hnRNP C₁ and C₂ proteins (Dreyfuss, 1986; Pinol-Roma et al., 1989) (Fig. 9) were all excluded from the PIKA regions. Similar results were observed for the hnRNP A₁ and L proteins (results not shown). These results were seen regardless of the order in which the antibodies were added (data not shown). The observed exclusion of these nuclear RNA-associated proteins was true for both the diffuse and concentrated forms of Sm (Nyman et al., 1986) and the hnRNP antigens (Pinol-Roma et al., 1989). These results suggest that both snRNP and hnRNP are found only rarely (or not at all) in the large PIKAs.

Discussion

PIKAs Are Large Subnuclear Domains of Unknown Function

PIKAs are large, roughly spherical nuclear regions that were first visualized after staining with a specific antibody, but that are also visible by phase-contrast and electron microscopy. Two arguments suggest that PIKAs represent a fundamental structural feature of the cell nucleus. First, they are visible both by phase-contrast and immunofluorescence microscopy after a variety of preparative procedures, including the use of three different fixatives (methanol, formaldehyde, or glutaraldehyde), as well as different wash and incubation buffers. Second, PIKAs were visible in all human cell lines tested (both transformed and primary cultures). This suggests that PIKAs are not a consequence of specialization within a particular cell type. PIKAs may have gone undiscovered before the development of the antibodies discussed in this paper because they have a lower density than the surrounding nucleoplasm.

We have thus far been unable to demonstrate PIKA-like nuclear staining in nonhuman cell lines. This may be a consequence of species specificity of the antibodies, since phase-light regions with a morphology similar to PIKAs could be visualized in these cells.

When nuclei are extracted with various combinations of detergent, nucleases, and high salt or chaotropic agents, the residual insoluble material is referred to as the nuclear matrix (Berezney and Coffey, 1977; Berezney, 1984; Kaufmann et al., 1986). The view of many researchers in this field is that the insoluble proteins of the nuclear matrix comprise a structural scaffolding for the nucleoplasm (Berezney, 1984; Vogelstein et al., 1985). PIKAs are sensitive to nuclease treatment in permeabilized cells and are therefore not part of the nuclear matrix.

Composition of the PIKAs

Rabbit antibodies that bind to the PIKAs also bind to the p23–25 proteins, suggesting that these proteins are concentrated within this nuclear compartment. However, human autoantibodies that also bind to these proteins on blots do not react with PIKAs by immunofluorescence. This is consistent with the results of earlier studies which failed to detect any significant binding to cultured cells with affinity-purified anti-p23–25 autoantibodies (Guldner et al., 1984; Earnshaw and Rothfield, 1985). We have demonstrated elsewhere that the human anti-p23–25 autoantibodies bind to a limited region of <58 amino acids on these proteins, and possibly to a single epitope within this region (Saunders, W. S., and W. C. Earnshaw, manuscript in preparation). It is therefore possible that the portion of the p23–25 proteins recognized by the autoantibodies is not accessible in the intact cell. While we cannot exclude the possibility that the anti-PIKA antibodies may cross-react in situ with an antigen other than the p23–25 proteins, it is important to note that affinity-purified rabbit antibodies that detect only the p23–25 antigens on immunoblots exhibit PIKA fluorescence identical to that seen with whole serum (Fig. 3, *J* and *K*).

Enzyme digestion experiments suggest that DNA is required for the structural integrity of the PIKA. PIKAs are

no longer detected by immunofluorescence after treatment of permeabilized cells with micrococcal nuclease or DNase I. Immunoelectron microscopy shows that the antigens are localized on ~ 15 -nm-diam fibers that traverse a less dense region of the nucleoplasm. These fibers may be chromatin in an extended configuration. The simplest interpretation of these results is that DNA is a structural component of the PIKAs, although other explanations are also possible.

Structure of the PIKAs Changes Dramatically during the Cell Cycle

When logarithmic cultures are stained with anti-PIKA serum, several different patterns of nuclear staining are observed. This variability in staining is correlated with the cell cycle phase. PIKAs of G_1 or S phase cells frequently comprise one or a very few large nuclear domains, each of which may be up to 5 μm in diameter. The presence of a discrete substructure within these domains is suggested by the speckled pattern of antibody binding. One interpretation of this morphology is that the larger PIKAs are aggregates of smaller subunits. This is consistent with the changes in PIKA structure seen during the cell cycle. As the PIKAs become progressively smaller and more dispersed, the larger structures apparently break apart into smaller clusters and eventually into single foci. These foci are about the size of centromeres stained with antibodies specific for CENP-B (Earnshaw et al., 1987).

Evidence from several laboratories suggests that interphase chromosomes occupy nonoverlapping domains within the nucleus (Manuelidis and Borden, 1988; Pinkel et al., 1988; Haaf and Schmid, 1991). The size of the larger PIKAs approaches the size of chromosomal domains of human lymphocytes (Pinkel et al., 1988) and it is possible that the large PIKAs observed during the first half of the cell cycle represent such chromosomal domains. This might explain the dependence of the immunofluorescence on intact DNA. Furthermore, if the PIKA proteins are associated with DNA, they may then serve as markers for the movement of underlying specific DNA sequences. The dramatic changes in PIKA morphology during late G_1 and S phases may be indicative of structural rearrangements that occur in the nucleus at this time. Examination of the distribution of centromere antigens as a function of cell cycle phase supports the notion that chromosomal domains undergo significant movements as a function of cell cycle phase (for review see Haaf and Schmid, 1991).

Possible Functions of the PIKAs in Cells

The observation that PIKAs correspond to less dense regions of the nucleoplasm suggests that the antigens are associated with euchromatin. This, in turn, could be consistent with a role for the PIKA proteins in either transcription or some facet of RNA processing or storage. We believe this to be unlikely, however.

There is increasing evidence for higher-order organization of RNA transcription and processing within the nucleus. This was first seen for RNA processing as a result of the availability of autoantibodies specific for components of the processing machinery. Three-dimensional reconstruction of anti-Sm-labeled cells from electron micrographs revealed

that snRNP antigens are distributed in a reticular network occupying $\sim 18\%$ of the nuclear volume (Spector, 1990). Smaller specialized areas of apparent spliceosome assembly (identified using a monoclonal antibody to an antigen designated SC-35) have also been identified (Fu and Maniatis, 1990). Discrete concentrations of hnRNP have been demonstrated with antibodies to the hnRNP-associated L protein (Pinol-Roma et al., 1989). In addition to being distributed throughout much of the nucleus, a subfraction of this protein was found to be concentrated in 1–3 large regions in all HeLa nuclei.

More recently, it has been shown that polyadenylated RNA is concentrated in ~ 20 – 40 discrete foci within the nucleus, which have been referred to as “transcript domains” (Carter, K., and J. Lawrence, personal communication). Because the transcript domains also correspond to major foci of Sm antibody staining, it is possible that significant levels of nuclear RNA processing also occur within these compartments.

Double staining for PIKAs and for Sm and hnRNP antigens reveals that the latter antigens are excluded from PIKAs. Thus, PIKAs are distinct from the transcript domains, and are unlikely to represent sites of RNA processing or storage. They are also unlikely to correspond to major loci of polymerase II transcription, since while RNA polymerase II does occur within PIKAs, this may simply reflect the distribution of the enzyme throughout the nucleus. Specific antibodies show no evidence for concentration of polymerase II in the PIKAs. Furthermore, we have failed to notice any change in structure of the larger PIKAs after prolonged exposure to the transcriptional inhibitor α -amanitin (Lindell et al., 1970; results not shown).

Together, these observations suggest that PIKAs represent areas of euchromatin that are neither sites of active RNA transcription, storage, or processing. The functional significance of the PIKAs thus remains unknown.

Compartmentalization within the Nucleus

The observations that some nuclear proteins (the p23–25 antigens) are specifically concentrated in the PIKA while others (snRNP- and hnRNP-associated proteins) are apparently excluded from it suggest that the PIKA is a distinct nuclear compartment. It is tempting to speculate that this segregation of protein antigens may be reflective of a broader pattern of functional segregation within the nucleus as well.

Until very recently, the concept that the nucleoplasm might be subdivided into discrete compartments was considered hypothetical, and was little supported by direct observation. The only nuclear substructures, other than the nucleolus, that had been described had been observed solely by electron microscopy and nothing was known about their constituents. This has now changed, however, and three well-documented examples of mammalian nuclear compartmentalization have been reported in the recent literature.

The nuclear substructures that most resemble PIKAs are termed coiled bodies. These are spherical objects, 0.3–0.5 μm in diameter, that were initially observed using a histochemical stain for RNA in the electron microscope (Monneron and Bernhard, 1969). Coiled bodies contain twisted fibrils (presumably proteinaceous) that are insensitive to DNase I treatment. A specific protein component of coiled bodies has recently been identified using a human autoanti-

body, in a study that reveals both significant similarities and differences between coiled bodies and the PIKA (Raska et al., 1991). The two resemble one another in four ways: (1) both show a frequent proximity to the nucleolus; (2) both contain a lower concentration of DNA than the surrounding nucleoplasm; (3) both are devoid of hnRNP L protein; and (4) both appear to lack interchromatin granules. Despite these similarities, PIKAs and coiled bodies show several differences. The size of the marker antigens is different (80 kD for coiled bodies and 23–25 kD for PIKAs), although this does not exclude the presence of both antigens in a single structure. More significantly, the number and size of the two structures differ. Antibody staining failed to reveal more than eight coiled bodies per nucleus (Raska et al., 1991), while a significantly larger number of PIKA foci is seen in the latter part of the cell cycle. In addition, the largest PIKAs ($\sim 5 \mu\text{m}$) are 5–10-fold larger than the largest coiled bodies. Finally, the appearance of the two structures (as observed by electron microscopy) differs. Coiled bodies are typically more dense than the surrounding nucleoplasm and are often surrounded by a capsule (Monneron and Bernhard, 1969). In contrast, the PIKA appears to be less dense than the surrounding nucleoplasm and lacks a defined capsule. We conclude that coiled bodies and PIKAs are most likely to be distinct entities within the nucleoplasm.

PIKAs show less resemblance to nuclear bodies, which are spherical, encapsulated structures that apparently bud off of the nucleolus and may contain nascent ribosomal RNA (Vagner-Capodano et al., 1982). The structure of nuclear bodies varies considerably: both the granular and fibrillar components of the nucleolus can be seen in different proportions for different nuclear bodies (Dupuy-Coin and Bouteille, 1972). Autoantibodies that recognize nuclear bodies have recently been described (Fusconi et al., 1991). These recognize an antigen of either 78–92 or 96–100 kD (it was not possible to distinguish between the two). Between 5 and 15 nuclear bodies could be detected per cell, and in all circumstances these were significantly smaller than the large PIKAs. Because nuclear bodies look even less like PIKAs by electron microscopy than do coiled bodies, we again conclude that the two are likely to be distinct structures.

A third nuclear substructure more similar to the PIKA was identified using an autoantibody that reacts with a 55-kD nuclear protein (Ascoli and Maul, 1991). This antibody recognizes a scattering of “nuclear dots” in growing cells from a variety of organisms. These dots resemble the PIKAs as they appear in the later parts of the cell cycle. Like the PIKA, nuclear dots are also apparently not associated with RNP protein domains or centromeres. However, they differ from the PIKA in two important regards. First, at no time in the cell cycle does the size of the nuclear dots approach that of the PIKA during G_1 phase. Second, the fluorescence of nuclear dots is unchanged after treatment of the nuclei with DNase I. Thus, the nuclear dots apparently comprise yet another class of nuclear domains that is distinct from the PIKA.

Together, these recent results are consistent with an emerging view of the structure of the nucleus. This view suggests that the nucleoplasm is, at least in part, differentiated into distinct structural domains. Some of these domains are visible by phase-contrast microscopy alone. Nucleoli are a conspicuous example of this type of compartmentalization. The PIKAs, while visible, are less striking under phase con-

trast and required the development of specific antibodies for their presence to be convincingly demonstrated. Careful examination of fixed cells by phase contrast reveals the presence of other large, structurally distinct “phase-light” nuclear regions that do not bind the anti-p23–25 antibodies. We suspect that these represent other specialized nuclear domains, and suggest that as antibodies for other nucleoplasmic proteins are developed, markers for these regions may become available as well.

Conclusions

These experiments, together with previous work describing the hnRNP L (Pinol-Roma et al., 1989), SC-35 (Fu and Maniatis, 1990), and “nuclear dot” antigens (Ascoli and Maul, 1991) support an emerging view of the nucleus as being compartmentalized into multiple discrete domains that can often be identified only using antibody probes. The most striking observation presented here is that the PIKAs (like nucleoli) undergo dramatic morphological alterations as cells traverse the cell cycle. It will be interesting to note whether other domains are static, or whether, like the PIKAs, they undergo structural rearrangements as a function of cell cycle phase.

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